T-Cell Clones With L3T4-Positive or Lyt-2-Positive Phenotypes Responding to Mutant MHC Class II Antigen and Inducing Graft Versus Host Reaction

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Two types of T cell clones responding to mutant major histocompatibility class II antigen (Ia^{bm12}) were established from spleen cells of C57BL/6 mice: one was L3T4-positive and the other Lyt-2-positive. These two types of clones carried functionally different properties. Lyt-2^+ clones were absolutely dependent on exogenous interleukin-2 for their proliferation, whereas some L3T4^+ clones secreted interleukin-2 and proliferated autonomously. Both types of clones had cytotoxic activities to bm12 target cells, and Lyt-2^+ clones showed stronger activities than L3T4^+ clones. Lyt-2^+ clones induced induration in situ, whereas the L3T4^+ clones induced ulcerative reaction when injected intradermally into mice. Histologically, the L3T4^+ clones caused necrosis of the epidermis or upperdermis, while the Lyt-2^+ clones induced infiltration of small round cells through the epidermis to the subcutaneous tissues and caused thickening of the epidermis. These characteristic reactivities might be due to a difference in lymphokines produced by each type of T cell subset in response to Ia^{bm12} antigen. *J Invest Dermatol 93:691–694, 1989*

Subsets of murine T lymphocytes responding to allogeneic major histocompatibility complex (MHC) class I and II antigens have been extensively studied and it seems well established that T cells with L3T4^-/Lyt-2^+ phenotypes selectively respond to MHC class I antigens, whereas those with L3T4^+/Lyt-2^- phenotypes respond to MHC class II antigens [1–7]. Thus, molecules on the T cell surface detected by either anti-Lyt-2 or anti-L3T4 antibodies are closely associated with the antigen-specific T cell receptor, and they are supposed to compose restriction elements for T cells to respond to MHC class I or II antigens [8], although exceptions have been reported [9–12]. For example, Golding and Singer [10] showed L3T4^-/Lyt-2^+ and L3T4^+/Lyt-2^- cytotoxic T lymphocytes [CTL] to be against MHC class II antigen. In this article, we report on the establishment of two different types of T cell clones responding to MHC class II antigen, i.e., L3T4^+/Lyt-2^- and L3T4^-/Lyt-2^+ cells. These two types of clones are shown to have different properties in terms of IL-2 dependence for their proliferation, CTL activities, and elicitation of local skin reactions.

MATERIALS AND METHODS

Mice Male C57BL/6 (B6) and B6.C-H-2^{bm12} (bm12) mice were used. In some experiments, B6.C-H-2^{bm1} (bm1) and BALB/c mice were also used. Bm12 mice were donated by Dr. H. Ishikawa, Department of Microbiology, Keio University School of Medicine, Tokyo, and bm1 mice were given by Dr. T. Nishizawa, Department of Dental Research, National Institute of Health, Tokyo. B6 mice were from the Jackson Laboratory (Bar Harbor, ME). These strains were bred and maintained at our animal facilities. BALB/c mice were purchased commercially (Charles River Japan, Atsugi, Kanagawa). All mice were housed in sterile cages in a room supplying filtered air and they were given pelletted food and chlorinated water ad libitum.

Mixed Lymphocyte Culture Mixed lymphocyte culture (MLC) was carried out as described [13]. For primary MLC, 5×10^5 responder cells were cultured with an equal number of irradiated stimulator cells for three days. During the last 20 h of culture, 0.5 μCi ^{3}H-thymidine was added. For secondary MLC, responder cells were cultured with stimulator cells for five days, and 5×10^5 collected cells were further cultured with an equal number of stimulator cells as was done in the primary MLC.

Induction and Assay of CTL For the induction of CTL, 4×10^5 B6 spleen cells were cultured with 2×10^5 stimulator cells and five days later cells were collected to assess cytotoxic activities. In secondary CTL induction, collected cells were cultured in the same condition as the primary CTL induction, but for four days. CTL were assayed against MHC class II antigen using lipopolysaccharide-activated spleen cells (LPS) blasts which were obtained by culturing spleen cells with 20 μg/ml of LPS (*Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI) for two days. These target cells were labeled with ^{51}Cr as described and activities of CTL were measured by ^{51}Cr-release assay [13].

Cloning T Cells T cells responding to MHC class II antigen (I-A^{bm12}) were grown in the culture containing the stimulator cells. Then, cloning of T cells was performed by a limiting dilution method in the presence or absence of Conconavalin A-activated rat spleen cell culture supernatant (Con A sup) as a source of growth factors including interleukin 2 (IL-2). Limiting dilution culture was repeated twice to obtain each single clone. Cloned T cells were

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Abbreviations:
CTL: cytotoxic T lymphocytes
Con A sup: concanavalin A-activated spleen cell culture supernatant
GVHR: graft-versus-host reaction
IL-2: interleukin-2
MHC: major histocompatibility complex
MLC: mixed lymphocyte culture
LPS: lipopolysaccharide

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further maintained in the presence or absence of Con A sup with weekly stimulation by irradiated stimulator cells.

Phenotypic Analyses of T Cell Clones Expression of Thy-1.2, L3T4, and Lyt-2 phenotype on cell surface was analyzed by the flow cytometer (Spectrum III, Ortho Diagnostic Systems, Westwood, MA). Thy-1.2$^+$ and Lyt-2$^+$ cells were detected by fluoresceinated anti-Thy-1.2 and anti-Lyt-2 monoclonal antibodies (Becton Dickinson, Mountain View, CA), respectively. L3T4$^+$ cells were detected by reaction first with rat monoclonal antibody to L3T4 and then with fluoresceinated anti-rat IgG antibodies (Medical Biological Laboratories, Nagoya, Japan).

Induction of Skin Reactions and Histologic Examinations Cloned cells were grown in 24-well culture plates with the stimulator cells for five to seven days. Viable cells from the cultures were collected by centrifugation over Ficoll-Conray solution to remove dead cells. The collected cells were thoroughly washed in minimum essential medium and injected at 1.5 – 10 × 10^6 intradermally at the depilated back skin of F1 hybrid or syngeneic mice. On day 3 or 5, a small piece of skin was excised from the cell-injected site or intact portion. Skin specimens were fixed in 10% formalin, dehydrated in graded alcohol and xylene, and embedded in paraffin. Sections of 4-μm thickness were made as usual and stained with hematoxylin and eosin.

RESULTS

In preliminary experiments, spleen cells from either B6 or bm12 mice were cultured with various sources of MHC class I- and/or class II-disparate stimulator cells. The primary and secondary proliferative and cytotoxic responses were assayed on the third day of culture. As reported [13], it was confirmed that both B6 and bm12 spleen cells were shown to respond to MHC class I and/or class II differences (data not shown). Because proliferative response of B6 anti-bm12 was constantly higher than that of bm12 anti-B6 in several experiments, B6 spleen cells were used as the responder in the following experiments.

Cloning of B6 T Cells Responding to bm12 Stimulator B6 T cells responding to bm12 stimulator cells were successively maintained in in vitro culture with or without rat Con A sup as a source of growth factors. Consecutively, T cells were cloned by a limiting dilution culture and maintained in microplate wells in the presence or absence of rat Con A sup. Twenty-four clones were established and their phenotypes were determined by flow cytometry. Cell surface phenotypes of representative clones are shown in Table I together with their proliferative and cytolytic activities. Phenotypes of clones were initially classified into three groups: L3T4$^+$/Lyt-2$^-$, L3T4$^-$/Lyt-2$^+$, and L3T4$^+$/Lyt-2$^+$. Flow cytometric patterns of three of the representative T cell clones are shown in Figure 1. The third group (clones BM-2 and BM-3) lost Lyt-2 phenotype gradually as passage of cells proceeded and was classified finally into the L3T4$^+$/Lyt-2$^-$ group.

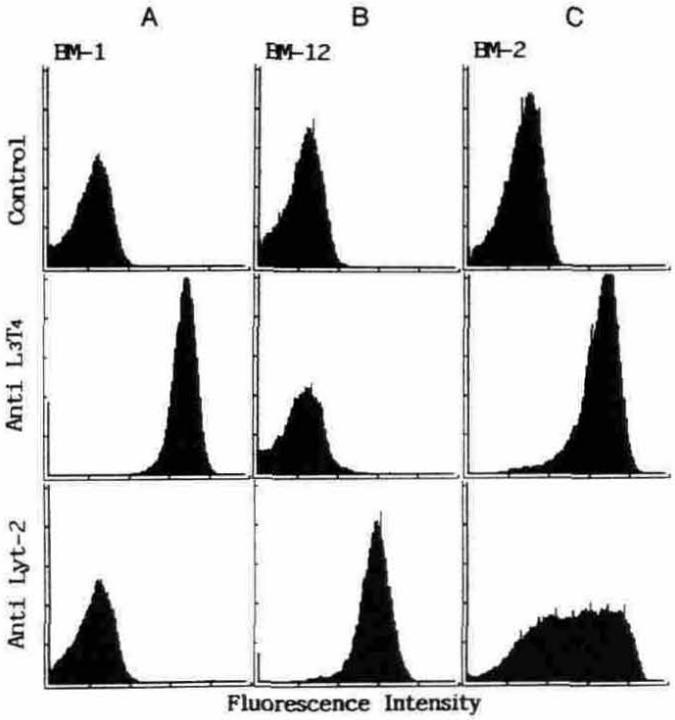

Figure 1. Analyses of cell surface markers of cloned T cells by flowcytometer. Typical patterns of *(A)* L3T4$^+$/Lyt-2$^-$ clone, *(B)* L3T4$^-$/Lyt-2$^+$ clone, and *(C)* double positive cells.

Functional Properties of T Cell Clones Most T cell clones were maintained in the culture medium containing 2.5 or 10% of rat Con A sup. L3T4$^-$/Lyt-2$^+$ clones became dependent on exogenous factors for their growth and lost proliferative capacity in the absence of rat Con A sup even in the presence of stimulator cells. L3T4$^+$/Lyt-2$^-$ clones were either dependent or independent on Con A sup. Growth of the dependent clones was accelerated in the presence of rat Con A sup and the independent clones produced IL-2 and proliferated in response to the stimulator cells (data not shown).

The cloned T cells carried cytotoxic activities against LPS blasts of bm12 spleen cells as shown in Table I. The spectrum of cytotoxic activities of L3T4$^+$ clones seems bm12-specific, whereas that of Lyt-2$^+$ clones is rather broad. The latter cloned lysed bm12 LPS blast strongly, but they apparently lysed third party BALB/c or bm1 LPS blasts.

Local Skin Reactions Induced by T Cell Clones Five T cell clones (L3T4$^+$: BM-1 and BM-115; Lyt-2$^+$: BM11, BM-12 and BM-13) responding to Ia were individually grown in in vitro culture and 1.5 – 10 × 10^6 collected cells were injected intradermally

Table I. Cell Surface Phenotypes and Activities of Representative Clones

T Cell Clones	Con A sup Added in Culture (%)	Cell surface Phenotype (%)[a] L3T4	Lyt-2	Proliferative Response Against (cpm × 10^{-3})[b] bm12	bm1	BALB/c	C57BL/6	Cytolytic Activity (%) Against (% Cr-release)[c] bm12	bm1	BALB/c	C57BL/6
BM-1		89.9	0.8	68.0	0.4	27.0	0.7	16.2	−3.4	7.5	−25.5
BM-115	10.0	99.0	0.6	12.5	0.5	0.2	12.0	23.1	−2.8	2.8	2.7
BM-11	2.5	1.6	97.9	2.4	0.2	0.4	0.3	44.2	5.1	−14.3	7.1
BM-12	2.5	0.7	78.0	1.2	0.2	0.6	1.0	51.2	11.1	34.6	−3.1
BM-13	2.5	0.7	97.6	0.6	0.2	0.2	0.2	52.1	11.1	26.8	2.5
BM-106	10.0	2.1	94.3	0.9	0.2	0.2	0.5	15.9	−6.8	−5.1	−5.1
BM-2		97.9	35.2 → 4.1[d]	56.1	1.0	16.0	1.0	53.9	−10.3	−5.0	−20.4
BM-3		93.2	21.3 → 12.8	52.4	0.3	5.8	0.9	30.6	8.6	−10.0	−24.5

[a] Each number indicates percentage of positive cells assessed by flowcytometry.
[b] 5 × 10^3 cloned T cells were cultured with 2 × 10^5 stimulator cells for three days. ^{3}H-thymidine incorporation during the last 20 h is shown in cpm.
[c] Percentage of ^{51}Cr-release. Effector : target ratio was 40 : 1.
[d] Percentage positivity of Lyt-2 phenotype decreased as passage of cells proceeded.

Table II. Elicitation of Local Skin Reactions by Cloned T Cells[a]

	Cloned T Cells Injected						
	L3T4⁺/Lyt-2⁻				L3T4⁻/Lyt-2⁺		
	BM-1			BM-115	BM-11	BM-12	BM-13
Recipients	8–10 × 10⁶	3 × 10⁶	1.5 × 10⁶	8–10 × 10⁶		8–10 × 10⁶	
(B6 × bm12)F1	6/6	4/4[b]	0/4	8/8	2/2	2/2	2/2
B6	0/2	0/2	NT[c]	0/1	0/1	0/1	0/1

[a] Cloned T cells were injected intradermally into depilated back skin and local reactions were observed. Positive mice/total mice tested are shown.
[b] These mice showed visible but very slight changes.
[c] Not tested.

into (B6 × bm12) F1 mice. The F1 mice injected with more than 8 × 10⁶ cells showed obvious skin reactions as summarized in Table II. The skin lesions showed different features according to the phenotypes of cloned cells injected. L3T4⁺ cloned cells (clone BM-1) induced marked redness with ulcer formation (Fig 2A), whereas Lyt-2⁺ cloned cells (clone BM-13) induced mainly induration of the skin (Fig 2B). The same type of skin reactions was observed in two or three separate experiments. Histologically, a large number of small round cells massively gathered in the dermis through subcutaneous tissue in the skin area corresponding to the ulcer of mice

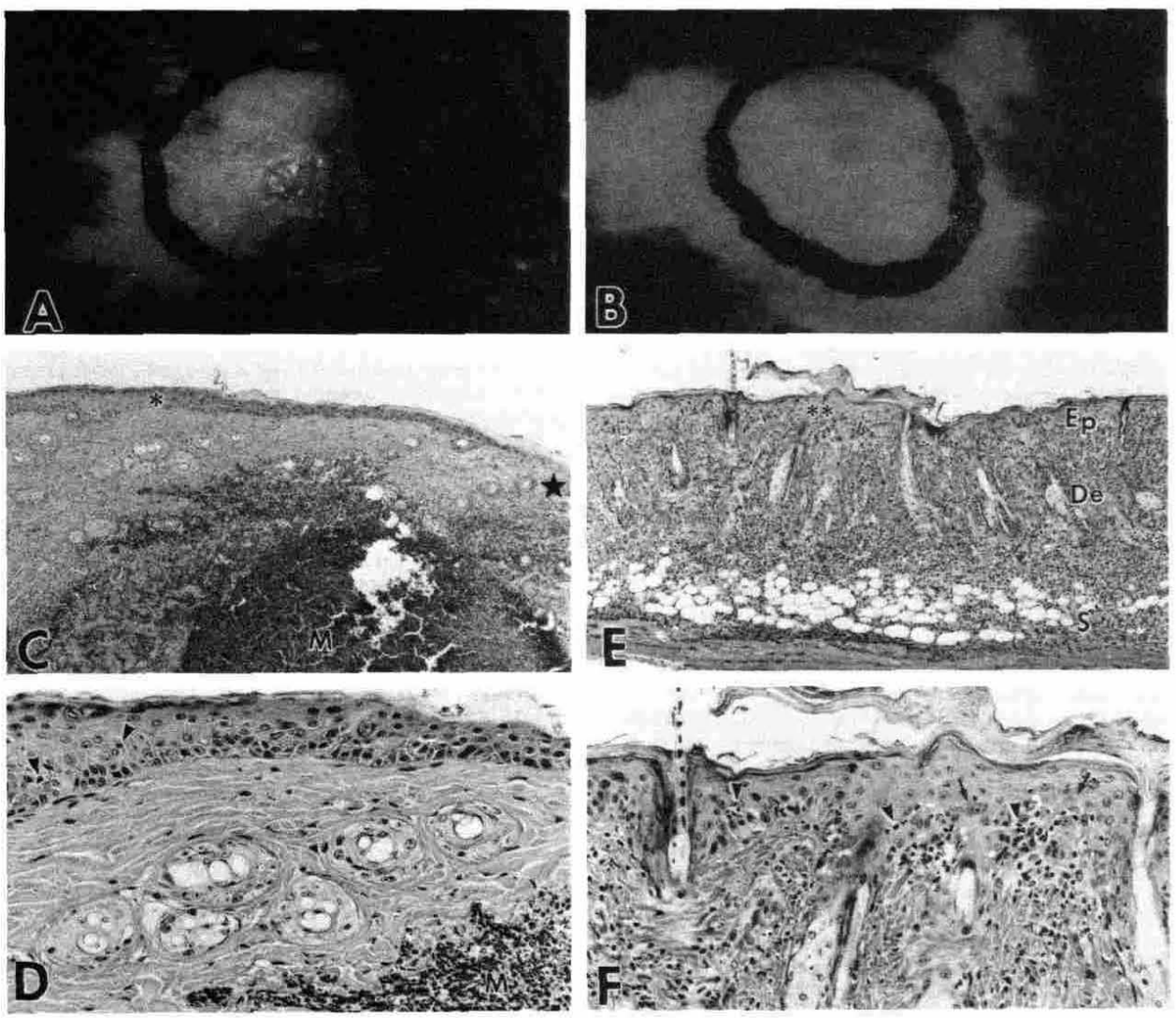

Figure 2. Skin reactions and histological findings. *A* and *B*, skin lesions induced by the injection of the clone BM-1 (L3T4⁺ cells) and the clone BM-13 (Lyt-2⁺ cells), respectively. Ulcer formation is seen in the center of the injected site indicated by the circle in *A* and an induration is present in the counterpart in *B*. *C* and *D*, histologic findings of the skin lesion shown in *A*. A mass *(M)* of a large number of small round cells is seen in the dermis through subcutaneous tissue. The covering epidermis and upper dermal tissue show necrosis in part *(star)*. The area indicated by an asterisk is enlarged in *D*. Although a few small round cells *(arrowheads)* are present in the epidermis, the epidermis shows normal thickness and no degeneration (H & E stain). *E* and *F*, histologic findings of the skin lesion shown in *B*. Small round cells are diffusely distributed in the lower epidermis *(Ep)* through the dermis *(De)* to the subcutaneous tissue *(S)*. The area indicated by double asterisks is enlarged in *F*. The epidermis shows thickening and many small round cells *(arrowheads)* are observed in the intercellular spaces of the lower epidermis. Some epidermal cells *(arrows)* become eosinophilic and the dermo-epidermal junction is obscure (H & E stain).

injected with L3T4$^+$ cells (clone BM-1). Almost the same histologic findings were obtained in the skin of six mice injected with clone BM-1 and also in eight mice injected with BM-115. The epidermis and upper dermis covering the mass of the cell infiltrate showed necrosis (Fig 2C). In the area surrounding the ulcer, a few small round cells infiltrated into the epidermis and dermis. However, neither degeneration nor thickening of the epidermis was seen (Fig 2D). On the other hand, small round cells diffusely infiltrated into the lower epidermis through the dermis to the subcutaneous tissue of mice injected with Lyt-2$^+$ cells (clone BM-13). This finding was entirely and evenly seen in the induration area of the skin (Fig 2E). The epidermis showed thickening and partial hyperkeratosis; many small round cells invaded the intercellular spaces in the basal to spinous cell layers of the epidermis, resulting in the presence of some eosinophilic necrotic epidermal cells and in the obscurity of the structure of the dermoepidermal junction (Fig 2F). These findings were confirmed in another five mice injected with Lyt-2$^+$ clones (two with BM-11, two with BM-12, and one with BM-13 clones).

DISCUSSION

A number of reports have shown that L3T4$^+$ T cells selectively react with MHC class II antigen, whereas Lyt-2$^+$ T cells respond to MHC class I antigens [1–7]. Exceptions to this rule have also been reported [9–12]. In this report, two types of T cell clones responding to MHC class II antigen were shown to be established: one with L3T4$^+$/Lyt-2$^-$ and another with L3T4$^-$/Lyt-2$^+$ phenotype. The L3T4$^-$/Lyt-2$^+$ type T cells responding to MHC class II antigen might not be a rare population, as four of 24 clones established belonged to this type. Golding et al [10] also reported on the T cells with Lyt-2 phenotype responding to MHC class II antigen. From these experimental results, it might be safe to conclude that Lyt-2$^+$ as well as L3T4$^+$ T cells are able to respond to MHC class II antigen. At the initial phase of this study, double-positive (L3T4$^+$/Lyt-2$^+$) T cell clones were established. Lyt-2 phenotype in these clones were labile; all of them lost the Lyt-2 phenotype as passage of the cells proceeded, and were grouped finally into L3T4$^+$ cells. The change of phenotype of T cells as culture generation proceeded was also observed in our previous report [13] in which T cell clones responding to MHC class I antigen were studied. Some clones gradually lost Lyt-2 phenotype while maintained in a medium containing a relatively higher concentration (20%) of rat Con A sup. In this study, L3T4$^+$/Lyt-2$^+$ clones were shown to lose the Lyt-2 phenotype even if maintained in the medium without rat Con A sup. They retained cytotoxic as well as proliferative activities against LPS blasts of bm12 spleen cells.

Two types of T cell clones had different properties in terms of IL-2 dependency or producibility. Lyt-2$^+$ and L3T4$^+$ T cells are both known to have an ability to produce IL-2 [14–16]. However, all of Lyt-2$^+$ T cell clones established in this study became obligatorily IL-2-dependent and lost IL-2 producibility during the successive culture in the medium containing Con A sup.

When Lyt-2$^+$ cloned T cells were injected intradermally into semiallogeneic recipients, they induced induration of skin with slight redness. This skin reaction was histologically similar to that seen in the graft-versus-host reaction (GVHR) induced by B6 Lyt-2$^+$ T cell clones recognizing mutant MHC class I antigen (H-2K^{bm1}) [13]. The main features were lymphocyte infiltration and structural disorder in the dermoepidermal junction. On the other hand, L3T4$^+$ cloned T cells evoked quite different histologic changes characteristic of massive necrosis. At present, it is not known why these two types of T cell clones induced different types of local GVHR or skin lesions. One possibility is that these two types of T cells might secrete different kind of lymphokines. L3T4$^+$ T cells are known to mediate delayed type hypersensitivity reaction and produce various lymphokines which activate macrophages [17,18]. Opposingly, Lyt-2$^+$ T cells mediate cytolysis and might secrete different type of lymphokines from L3T4$^+$ T cells. These problems are under investigation at our laboratory.

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